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Matrix Vesicle Enzyme Activity and Phospholipid Content
in Endosteal Bone Following Implantation of Osseointe-
grating and Non-Osseointegrating Implant Materials

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Matrix Vesicle Enzyme Activity and Phospholipid Content in

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Non-Osseointegrating Implant Materials

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THESIS

Presented to the Faculty of

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MASTER OF SCIENCE

By

Thomas S. Marshall, B.S., D.D.S.
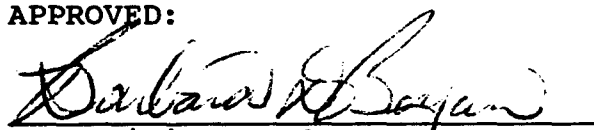

San Antonio, Texas

November, 1991

MATRIX VESICLE ENZYME ACTIVITY AND PHOSPHOLIPID CONTENT IN
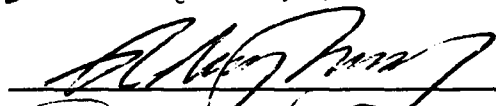ENDOSTEAL BONE FOLLOWING IMPLANTATION OF BONDING AND NON-BONDING
IMPLANT MATERIALS

Thomas Scott Marshall

APPROVED:

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Supervising Professor

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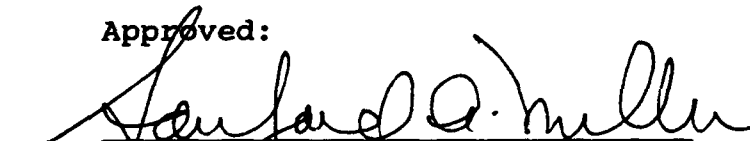
November 13, 1991
Date

Approved:

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Sanford A. Miller, Ph.D.
Dean

DEDICATION

I wish to dedicate this work to my parents, Joseph J. and Ruth E. Marshall. Their unswerving dedication to raising four children made it possible for me to be what I am today. Through care, love, and compassion, they helped mold my perspective on the world and life in general. This is a debt that a child can never repay. All I can say is THANK YOU!

ACKNOWLEDGMENTS

I would like to take this opportunity to thank the members of my research committee, the orthopaedic research laboratory and the staff of the Industry/University Cooperative Research Center, without whose help this project could not have been performed. In particular, I would like to thank the chairperson of my research committee, Dr. Barbara D. Boyan, for her faith in my abilities, her encouragement, and her willingness to show clinicians that there is a place for research in our lives. Special note must go to Dr. Zvi Schwartz for his friendship and exemplary dedication to research and the advancement of Dentistry. His planning, support, and guidance made this project possible. To Dr. Larry D. Swain, I give thanks for his calm and realistic views to subdue the turmoil that follows in the wake of all research. Thanks go to Drs. Cronin and Morrow for being willing to look at the unknown and assist in a project that was new to all of us. This project could not have been completed without the help of Ms. Virginia Ramirez, whose assured belief that there really was something at the bottom of the tubes allayed much trepidation, and Ms. Sandy Messier for her timely preparation of this and other manuscripts.

Matrix Vesicle Enzyme Activity and Phospholipid Content in
Endosteal Bone Following Implantation of Osseointegrating
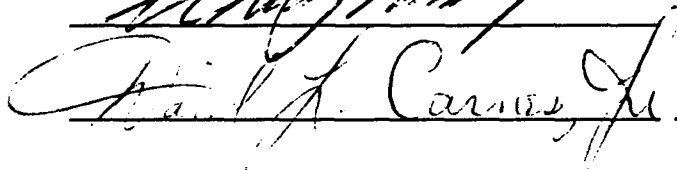and Non-osseointegrating Implant Materials

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Supervising Professor: Barbara D. Bcyan Ph.D.

To date, little has been done to standardize testing
procedures of implant materials for use in human prostheses.
This series of experiments is part of an extensive study dealing
with primary mineralization at the interface of osseointegrated
and non-osseointegrated implant materials. The importance of
this experiment is to ascertain the ability of a variety of
materials to osseointegrate with bone, utilizing assays of matrix
vesicle enzyme activity and lipid content. The process of
osseointegration is dependent upon the chemical composition of
the implant material, the physical properties of both the implant
material and bone, the clinical/surgical procedures utilized, and
any forces placed at the bone/implant interface. The surgical
procedures and collection of marrow samples were performed prior
to the initiation of the biochemical analysis. Four different
implant materials were utilized (KG-Cera, Mina 13, KGy-213,

M 8/1) and evaluated for their effect on normal bone repair. Prior to placement of the implants, the tibial marrow space of the treatment leg was ablated. Evacuation of the marrow cavity prior to implant placement has been shown to activate endosteal bone repair. Five hundred seventy-two separate implants were surgically placed in the proximal tibial bone of 572 male albino rats. Thirty-six additional animals were utilized as a nonsurgical time "0" control, thus bringing the total experimental population to 612. The implanted animals were sacrificed on days 3, 6, 14, and 21, with subsequent curettage of the bone marrow chambers from the implant and contralateral tibias. The marrow from both tibias was collected to compare the matrix vesicle enzyme and lipid activities in both the treatment and contralateral limbs. A previous study (Schwartz et al., 1989) has demonstrated that increased activity is seen in both limbs following surgery, with a significantly greater response noted in the treated limb. The collected hemopoietic material was a combination of cells, fractured cell debris, and matrix vesicles. This material was utilized for the following experiment:

a. Differential centrifugation was used to isolate matrix vesicles from the marrow samples. It is believed that the matrix vesicles are an initial locus for calcification in most calcified matrices.

b. The studies to be performed include: alkaline phosphatase specific activity, phospholipase A_2 specific activity, phospholipid composition, protein content, and

succinic-INT-reductase, which is a check for the presence of mitochondrial contamination.

It has been proposed that biochemical analysis of enzyme activity and lipid composition can be a valuable adjunctive test for bone formation, mineralization, and the physiological response of mineralized tissue to different implant materials. The parameters outlined in this experiment could be utilized in a standardized criteria for the evaluation of implant materials and osseointegration.

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I. INTRODUCTION

The application of biologically compatible osseointegrating materials continues to have an increasing impact on oral health care. A thorough understanding of primary mineralization is required in order to select materials that will complement the biological system and function as support for future prostheses. The utilization of osseointegrated implants has shown a steady increase over the last 20 years, with applications in fixed, removable, and maxillofacial prostheses. Predicted future demographics denote an ever-expanding role, both in utilization and application of these devices. Predictability of tissue response and outcome are important both for the clinician and the patient.

Osseointegration of implant materials can have a significant effect on retention and function of prostheses. Osseointegration is dependent upon the chemical composition of the implant material, the physical properties of both the implant material and bone, the clinical/surgical procedures utilized, and any forces placed at the bone/implant interface. The concept of osseointegration originated from vital bone marrow studies, in which a close association was seen between bone marrow and bone repair (Branemark, 1983). According to Dr. Branemark, osseointegration is a direct structural and functional connection between ordered living bone and the surface of the load-carrying implant (Branemark et al., 1985). In the 1960's, Branemark and associates found that titanium optical chambers with a screw-

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shaped design could not be removed from the adjacent bone due to ingrowth of bone into the thread spaces. It was later found that the chemical properties at the interface of most metallic implants are determined by an oxide layer and not the metal itself (Kasemoto, 1983). Bioactive glass-ceramic bonding materials studied by Hench and associates (Hench et al., 1971, 1973, 1974) in the early 1970's demonstrated a silicon-rich layer high in calcium and phosphate ions in intimate contact with the bone. Light microscopy (LM) and transmission electron microscopy (TEM) have shown that the direct bonding of bone to bioglasses and other glass ceramics was due to the deposition and mineralization of an organic bone matrix composed of collagen and mucopolysaccharides at the outer layer of the implant (Clark et al., 1976). Some controversy exists today in that selected observers have reported an appearance of biointegration, where there is direct biomechanical bone/implant attachment with hydroxyapatite-coated implants . However, this assertion has not proven to be the case at the electron microscopic level, where a 20-150 angstrom layer of ground substance is noted separating the bone and implant interface (Hansson et al., 1983; Albrektsson and Hansson, 1986).

Non-osseointegrating implant materials are characterized by a nonmineralized fibrous tissue layer located at the bone/tissue interface. This tissue layer results in inadequate retention and deprives the bone of stimuli required for remodeling and maintenance (Branemark, 1983). The lack of mineralization has been attributed to a variety of conditions. In particular, a

variety of metals have been incorporated into the composition of implant materials to decrease their solubility to extracellular fluids. Aluminum is known to inhibit the formation of calcium phosphate crystals *in vitro* (Meyer, 1977), while aluminum, tantalum, and zirconium oxides have inhibitory effects on mineralization (Hench and Wilson, 1984; Gross et al., 1985; Blumenthal and Posner, 1984). Pure metallic titanium has also been shown to interfere with mineralization (Blumenthal and Cosma, 1989). Fibrous capsulization around the implant has been well-documented if functional loads or minor movement is applied to the implant during healing (Brunski et al., 1979). Nonbiologic prosthetic materials frequently utilize bone cements (methyl methacrylate) to anchor fixtures in place. This procedure results in the destruction of osteocytes at the interface and subsequent development of a poorly retentive soft tissue scar (Branemark, 1983).

The purpose of this experiment was to determine whether the biochemical characteristics of extracellular matrix vesicles during primary mineralization would be altered following placement of osseointegrating and non-osseointegrating implant materials. This experiment was the final segment of a combined experiment utilizing morphometric and biochemical analyses to evaluate the role of extracellular matrix vesicles as a diagnostic tool for implant assimilation. It has been proposed that a combination of biochemical and quantitative morphometric studies based on light and electron microscopic observations could be a diagnostic test for bone formation and mineralization

at the bone/implant interface. The reduction in time and specific observed parameters as outlined represent a superior method of evaluating the osseointegrating ability of new and existing implant materials. There has been considerable confusion in the literature as to the exact role of extracellular matrix vesicles in primary mineralization. Each implant material produces material-specific effects on the healing bone, as well as systemic effects to the body in general. Thus, perturbations of bone formation due to the implants and resulting effects on matrix vesicle biochemistry have provided an excellent model for sorting out issues concerning matrix vesicle structure and function. This study offers potential benefits for better understanding and improving materials and techniques in all mineralized tissues. It is expected that the criteria established may serve as a future standard for newly developed implant materials. The combination of automated image analysis, quantitative electron micrographic analysis, and biochemical analysis has not been utilized for evaluation of osseointegration prior to this time.

II. **LITERATURE REVIEW**

A. Implants and Osseointegration

The earliest example of osseointegration was found in an ancient Nubian burial site, which is presently covered by the waters of the first Aswan Dam in Egypt. The implant is an iron arrowhead located in the mid-body of the mandible of a Nubian woman between 18 and 20 years of age. The women of this tribe were known for their ferocity and support of their men in battle, so the appearance of a wound of this nature was not uncommon. However, the remodeling of the bone and what appears to be intimate contact around the arrowhead establishes this as a successful accommodation by the body to a foreign object (Harris, 1978).

The successful utilization of implant materials requires an in-depth knowledge of the composition of the materials to be used, their configuration, and the surgical procedures utilized in their placement. The ideal implant should react to physical stimuli and interface with the host tissue as if it were equivalent to the tissue it replaced (Gross et al., 1981). Materials designed for intraosseous implantation are required to follow several criteria: they should be nontoxic- and nontumor-inducing, biochemically stable, adaptable to function, have an absence of discomfort, promote normal bone growth and development, and be durable (Schnitman, 1979; Albrektsson et al., 1986). Metals, plastics, and bioceramics are presently the three

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most commonly used materials for biomedical implants (Hansson, 1983) and can be divided into two categories: osseointegrated and non-osseointegrated.

Bone tissue response to implant materials has been evaluated by employing histological techniques, morphometrical methods, and evaluation of serial sections of bone (Gross et al., 1977, 1980). The morphometric quantification of tissue components (bone, osteoid, chondroid, and soft tissue as a percentage of the implant interface) has provided a preliminary basis for the comparison of different implant materials. The animal model for implant study has been standardized for comparative purposes using the same implantation site in the diaphysis of the femur of male Sprague-Dawley rats. The results from this model have proven to be transferrable to a variety of other models and sites: maxilla and mandible of pigs (Strunz et al., 1982), the femurs of dogs and rabbits (Deutscher et al., 1978; Fuchs et al., 1981), and the mandibles of humans (Bunte et al., 1977).

Following surgical implantation, there is a specific healing sequence which should progress in order to get successful osseointegration. The implant should be placed in a prepared, blood-filled space to allow the formation of an organized blood clot in intimate contact with the material. The blood clot is then transformed by polymorphonucleocytes, lymphoid cells, and macrophages, allowing for the formation of a fibroblastic procallus. The dense connective tissue is then infiltrated by mesenchymal cells, which will differentiate into osteoblasts. The osteoblasts form an osteogenic matrix and transform the dense

connective tissue into a woven bone callus, which then progresses to mature bone through remodeling (Hobo et al., 1989).

Bone development and osseointegration are controlled by osteoblasts in the implant area after the disappearance of macrophages. Osteoblasts are responsible for the secretion of osteoid, which is composed of collagen and proteoglycans, as well as the production of extracellular matrix vesicles. The factors favoring osteoblastic differentiation are: 1) a bioactive implant material, 2) minimal tissue trauma, 3) a tight implant fit, 4) elimination of loading during healing, 5) the absence of infection during healing, 6) sufficient levels of bone-regulating substances, and 7) proper health and hygiene.

B. Primary Mineralization:

The theory of primary mineralization designates the initiation of hydroxyapatite formation as a major role of matrix vesicles (Anderson, 1969; Bonucci, 1970). It is thought that rupture of the vesicle membrane is a result of crystal growth. Once released to the matrix, crystals can adhere to each other to form calcospheritic structures, with secondary nucleation guided by the collagen. Electrically charged crystalites align alongside collagen fibrils and become incorporated into "hole sites," from which calcifying fronts are formed (Wuthier, 1982). Direct evidence of the step-by-step process of mineralization has recently been reported in the tibias of rats (Amir et al., 1988a, 1988b; Schwartz et al., 1987; Sela et al., 1987a, 1987b, 1987c).

When mineralization progresses adjacent to an implant, the osteoblasts become evenly distributed and arranged with their

long axis parallel to the surface of the implant. Collagen filaments are arranged 1-3μm from the implant surface and are perpendicular to the implant/bone interface. The filaments are separated from the implant surface by a 20nm-thick layer of glycoproteins (Hansson, 1983). The morphological basis for the biochemical quality of osseointegration has been demonstrated through TEM studies at the interface of implant materials fabricated from glass-ceramics and other materials. The progressive development of mineralized structures adjacent to osseintegrating implants is consistent with accepted concepts of primary bone formation (Boyde, 1969; Owen, 1971; Anderson, 1976a; Simmons, 1976; Sela et al., 1978a; Gross et al., 1981; Sela et al., 1981).

Transmission electron microscopic and computerized morphometric studies have been used to examine changes in extracellular matrix vesicles during the first three weeks of endosteal healing following an injury (Sela, 1987b). The bone injury included removal of the marrow, followed by regeneration of the tissue via primary mineralization. The extracellular matrix vesicles were examined on days 3, 6, 14, and 21 following injury. Most of the vesicles were found less than 3 μm from the calcification front, with ruptured vesicles located closest to the front. As healing progressed, vesicles of all types decrease in diameter as well as distance from the calcification front.

An additional study examined whether the morphologic appearance of matrix vesicles and initial formation of internal crystals could be correlated to changes in phospholipid

composition and metabolism (Wuthier, 1975). The morphologic appearance of the matrix vesicles was assessed by morphometric analysis. In parallel experiments, matrix vesicle-enriched membranes (MVEM) were isolated from homogenates of endosteal tissue removed from treated tibias, as well as from the contralateral control. There was an increase at six days in MVEM alkaline phosphatase and phospholipase A_2 specific activities in both limbs. The magnitude of response was significantly greater in the treated leg. There appears to be a systemic effect of marrow ablation that influences specific cellular metabolism at sites distant from the site of injury (Schwartz et al., 1989)

Research in the role of extracellular matrix vesicles in normal and pathological mineralization is receiving increased attention. A recent study on alveolar bone healing found that, following tooth extraction, there is an immediate decrease in the number of vesicles and their enzymatic activity. The healing process is later accompanied by cellular proliferation and production of active vesicles (Muhlrad et al., 1981). Originally, isolation of matrix vesicles to assess vesicular occurrence and activity was performed by enzymatic digestion and differential centrifugation. This method often yielded a matrix vesicle fraction contaminated with cellular organelles. To further purify the vesicle fraction, sucrose density gradients have been used for fractionation of both bone and cartilage preparations (Wuthier et al., 1978; Kahn et al., 1978; Deutsch et al., 1982; Muhlrad et al., 1983).

C. The Extracellular Matrix Vesicle

In 1967, Anderson and Bonucci (Anderson, 1969; Bonucci, 1971) first provided transmission electron microscopic evidence of extracellular matrix vesicles in calcifying epiphyseal cartilage through TEM studies. The vesicles appear to be round-to-ovoid in shape, vary in size from 300 to 2500 angstroms, and have a trilaminar membrane structure. Extracellular matrix vesicles have been shown to participate in the primary mineralization of bone, cartilage and dentin (Anderson et al., 1976b; Boskey, 1981; Wuthier, 1982; Ali, 1983). These extracellular organelles have also been associated with mineralization in a number of additional normal and pathologic conditions, including cementum (Hayashi, 1985), fracture callus (Boskey et al., 1980), reparative dentin (Bab et al., 1982), atherosclerosis (Tanimura et al., 1983), aortic valve calcification (Kim, 1978), osteosarcoma (Muhlrad et al., 1978), and renal calcifications (Ganote et al., 1975).

Membrane-associated mineralization is nonspecific to eucaryotic cells alone. A variety of procaryotic cells have demonstrated microbial mineralization via membranes. An example of this is the formation of dental calculus by colonies of organized dental plaque (Boyan et al., 1982; Ennever et al., 1978b; and Sidaway, 1980).

Although first thought to be an artifact of tissue preparation, some controversy still remains as to the exact origin of matrix vesicles. We will present the current theories

of origin, starting with the most to the least accepted. They are as follows:

a.   Bonucci (1970) established the spherical nature of the matrix vesicle, with Cecil and Anderson (1978) demonstrating the formation of extracellular matrix vesicles via budding from the plasma membrane of chondrocytes.   Since that time, budding has also been demonstrated in osteoblasts and odontoblasts.   Although the mechanism has not been well-described, it has been supported by transmission electron microscopic studies (Borg et al., 1978; Cecil and Anderson, 1978; Anderson, 1978; Glaert and Mayo, 1977). The data indicate that cell surface microvilli may be the precursor of the matrix vesicle.   Cell culture studies of chondrocytes indicate that matrix vesicles formed in culture are similar to vesicles formed *in vivo* (Boskey, 1978).   Similar phospholipid   contents,   particularly   with   respect   to phosphatidylserine, are noted for vesicles formed *in vivo* and *in vitro* (Wuthier, 1975; Peress et al., 1974; Boskey and Posner, 1977; Boskey, 1978).

b.   A second theory states that matrix vesicles are derived as a result of cell degeneration.   The presence of matrix vesicles associated with degenerative pathologic calcifications like atherosclerosis (Kim, 1976; Tanimura et al., 1983) and the increased number of vesicles found in ageing cartilage tissue (Bonucci and Dearden, 1976) are cited as possible support for the theory.   Biochemical analysis would tend to support the budding theory rather than cell degeneration in that matrix vesicles have a variety of plasma membrane marker enzymes and phospholipid

profiles that are unique to the matrix vesicles themselves (Wuthier, 1975; Bonucci, 1970). Simple degeneration of the membrane structure would produce similarity, rather than dissimilarity, between the plasma and the matrix vesicle membranes.

c. The third theory states that matrix vesicles are preformed by the intracytoplasmic membrane (Slavkin, 1972) and then extruded.

In the extreme diversity and complexity of the mineralization processes, any or all of these processes may be utilized. It has been proposed that matrix vesicles are not a homogeneous, but a heterogeneous population of organelles, where production may be cell-specific, come from multiple subclasses of matrix vesicles, or a combination of both. Further research is necessary to clarify this area.

D. Morphometric Analysis:

Extracellular matrix vesicles have biochemical properties that promote the initiation of crystallization (Ali, 1983). Sela et al. (1987) first demonstrated the four steps of vesicular crystal formation using qualitative computerized morphometry. Following release from the plasma membrane, the matrix vesicle appears empty, with an electron lucent content. This is followed by amorphous vesicles with a uniform electron opaque content due to loading with calcium and phosphate from extracellular fluids. Calcium and phosphate compounds are concentrated within the vesicle, and calcium-phospholipid complexes (CPLX) are formed. The crystal stage is characterized by the presence of an intravesicular nidus of calcification on the internal surface of the vesicle membrane. As mineral deposition continues, there is a formation of hydroxyapatite crystals within the vesicles. Normally there is a single crystal within an individual vesicle, but on occasion, multiple crystals are noted. The crystals are located either in the equatorial position or are incorporated within the boundary membrane. The final stage following crystal growth is rupture of the vesicle membrane and release of the contents to the extracellular environment (Ali, 1983). In the matrix, released crystals adhere to each other and serve as foci for continued crystal deposition. Calcospheric structures result, completing the first stage of primary mineralization. Matrix vesicles in and of themselves do not migrate to the mineralization front following extrusion from the plasma membrane, but in actuality remain stationary and form the nidus

for the mineralization front. Most matrix vesicles are found within 3μm of the mineralization front, with the ruptured vesicles being the closest and having the largest diameter. It must be emphasized that mineralization is a very complex and integrated process and that calcification by extracellular matrix vesicles is not the exclusive mechanism for mineralization in vertebrates (Glimcher and Krane, 1968; Hohling et al., 1981).

Quantitative morphometric analysis has been utilized to analyze changes in matrix vesicle structure and function during wound healing (Bab et al., 1983). A direct correlation was found between size, density, and rupture of matrix vesicles and mineral deposition along the calcification front. Electron-lucent matrix vesicles produced the highest levels of enzyme activity as compared to electron-dense vesicles (Muhlrad et al., 1981). Corrosion of the surface of implant materials has been shown to interfere with osseointegration. Leaching of material by extracellular fluids and the appearance of phagocytic cells with vacuoles containing ceramic particles (Gross et al., 1981; Sela et al., 1981) affect the long-term stability of the implant. Metal oxides incorporated into the implant materials stabilize solubility, but several, such as titanium, tantalum, and aluminum, have been shown to inhibit matrix maturation, mineral content, and primary mineralization (Gross and Strunz, 1980; 1982; 1985; Papas and Cohen, 1968; Gross, 1988; Blumenthal et al., 1988).

Alkaline phosphatase was the first vesicular enzyme discovered and has been established as having a critical role in

mineralization and bone formation (Ali, 1980; Majeska and Wuthier, 1975; Sela and Bab 1979a, 1979b). It is considered the marker enzyme for matrix vesicles. The exact role of alkaline phosphatase is unclear, but it has been found to promote hydrolysis of phosphate-containing substrates into orthophosphate. Orthophosphate is essential for crystal formation within the matrix vesicle (Ali 1973, 1976, 1983; Ali et al., 1977; Feliz and Fleisch, 1976; Hsu and Anderson, 1978). Alkaline phosphatase promotes the uptake of calcium by matrix vesicles and blocks the inhibitory effects of ATP on hydroxyapatite formation (Ali and Evans, 1981).

Phospholipase A_2 is another enzyme associated with matrix vesicle activity and is seen to increase along with alkaline phosphatase levels. The precise function of this enzyme on primary mineralization is unknown (Wuthier, 1975). Its role is felt to be in the disruption of the matrix vesicle membrane during the "rupture" phase of development by altering the phospholipid content and fluidity (Wuthier et al., 1978). Studies by Boyan and Schwartz (1988) have shown that phospholipase A_2 may be regulated by vitamin D metabolites, resulting in changes in phospholipid composition and content and permeability of the vesicle membrane.

Phospholipids are a significant component of the matrix vesicle membrane and are directly associated with mineralization of cartilage, bone and dentin (Shapiro, 1970; Dirksen and Marinetti, 1970). Matrix vesicles have been shown to have lipid compositions distinct from the plasma membrane (Boyan et al.,

1989; Peress et al., 1974; Boyan-Salyers et al., 1978). The composition of phospholipid has been shown to be related to the their role in mineralization (Wuthier, 1975). *In vitro* experiments have shown that phospholipid liposomes can act as sites of apatite formation (Canes and Hailer, 1987). Phosphatidylserine (PS) is a major component of the matrix vesicle membrane proteolipids (Boyan-Salyers et al., 1978) and calcium-phospholipid complex (CPLX) (Wuthier and Gore, 1977) and is the most closely associated with the mineral phase. CPLX has been shown to promote *in vitro* calcification in calcifying bacteria (Boyan-Salyers and Boskey, 1980) and animals (Boskey and Posner, 1977).

E. Normal Healing:

A recent study by Schwartz et al. (1989) examined the role of extracellular matrix vesicles during normal healing of rat tibial bone. Ninety male rats of the Hebrew University (Sabra) strain, weighing 400g each, were used in the experiment. The rats were divided into five groups of 18 animals. Eighteen animals were placed in a nonsurgical control group (time 0--no surgery was performed on either leg prior to marrow collection), while the remaining 72 animals were equally distributed in the surgical model.

Infrapatellar surgical incisions were performed under Ketanest/Rompun anesthesia (25mg--5mg/Kg body weight) to achieve access to the proximal aspect of the right tibia (no surgery was performed on the contralateral tibia prior to marrow collection). Entrance to the marrow chamber was achieved by frontal

penetration of the antero-medial aspect of the exposed bone with a saline-cooled, round dental burr (#4) and a 20,000 RPM motor. The bone marrow chamber was evacuated by repeated washings with saline through a cannula introduced into the intrabony space. Following ablation, individual samples of the glass-ceramic materials were introduced into the right tibia marrow space through the previously mentioned portal, followed by surgical closure of the access site. The animals were allowed to resume normal weight-bearing activities for 3, 6, 14, and 21 days.

At harvest, bilateral tibial excisions were performed on both experimental and control animals (day 0) under Ketanest/Rompun anesthesia (25mg--5mg/Kg body weight). The contents of each marrow space were carefully evacuated by curettage under a constant flow of saline and placed in individual containers. The tissue from subgroups of six rats each was pooled for further biochemical analysis. In addition, the contralateral limb was excised to examine tissue reaction distal to the primary site of injury. Studies by Gerasimov and Chailakhyan (1978), and Bab et al. (1985) intimate that trauma produces systemic effects similar to that seen at the primary site of healing.

Alkaline phosphatase specific activity increased from day 3 to day 14, with a peak at day 6 (Figure 1). The level of enzyme activity in the contralateral leg paralleled that of the treatment leg only at a significantly reduced level. By day 21, the enzyme activity in both the treatment and contralateral legs had returned to or near the baseline.

Phospholipase A_2 was also evaluated, with significant increases noted starting on day 3, peaking on day 14 and, remaining slightly above that of the baseline in the treatment limb on day 21 (Figure 2). As was previously observed with alkaline phosphatase, phospholipase A_2 activity levels in the contralateral limb mimicked those of the treatment limb, but fell to baseline on day 21.

Time-dependent variation was also noted in the phospholipid content of the matrix vesicles, particularly in sphingomyelin, which peaked at day 3, with a three- to four-fold increase over the baseline, decreasing to baseline by day 14 (Figure 3). Elevations in phosphatidylserine and cardiolipin were noted on days 6 and 14, with a return to baseline on day 21 (Figure 3). Phosphatidylcholine content was highest on day 0, with a subsequent 50% reduction on day 14. The levels did not return to baseline by day 21 (Figure3). As with the two previous studies, the contralateral leg paralleled that of the treatment leg (Figures 3 and 4). Phosphatidylserine levels for the treatment limb were significantly different from those of the contralateral limb on days 6 and 14 (Figures 3 & 4), whereas sphingomyelin and cardiolipin values were comparable for both preparations.

Figure 1. Specific Activity of Alkaline Phosphatase in Matrix Vesicle-Enriched Membranes (MVEM) Isolated from Healing Endosteal Bone following Marrow Ablation.

MVEMs were isolated by differential centrifugation of homogenates of newly forming bone in the treated and contralateral tibias. Values are the mean $\pm$ SEM for three samples. Each sample represents the pooled tissue of six treatment or contralateral legs. *p ~ 0.05 for each time point compared to time 0.

FIGURE 1. ALKALINE PHOSPHATASE
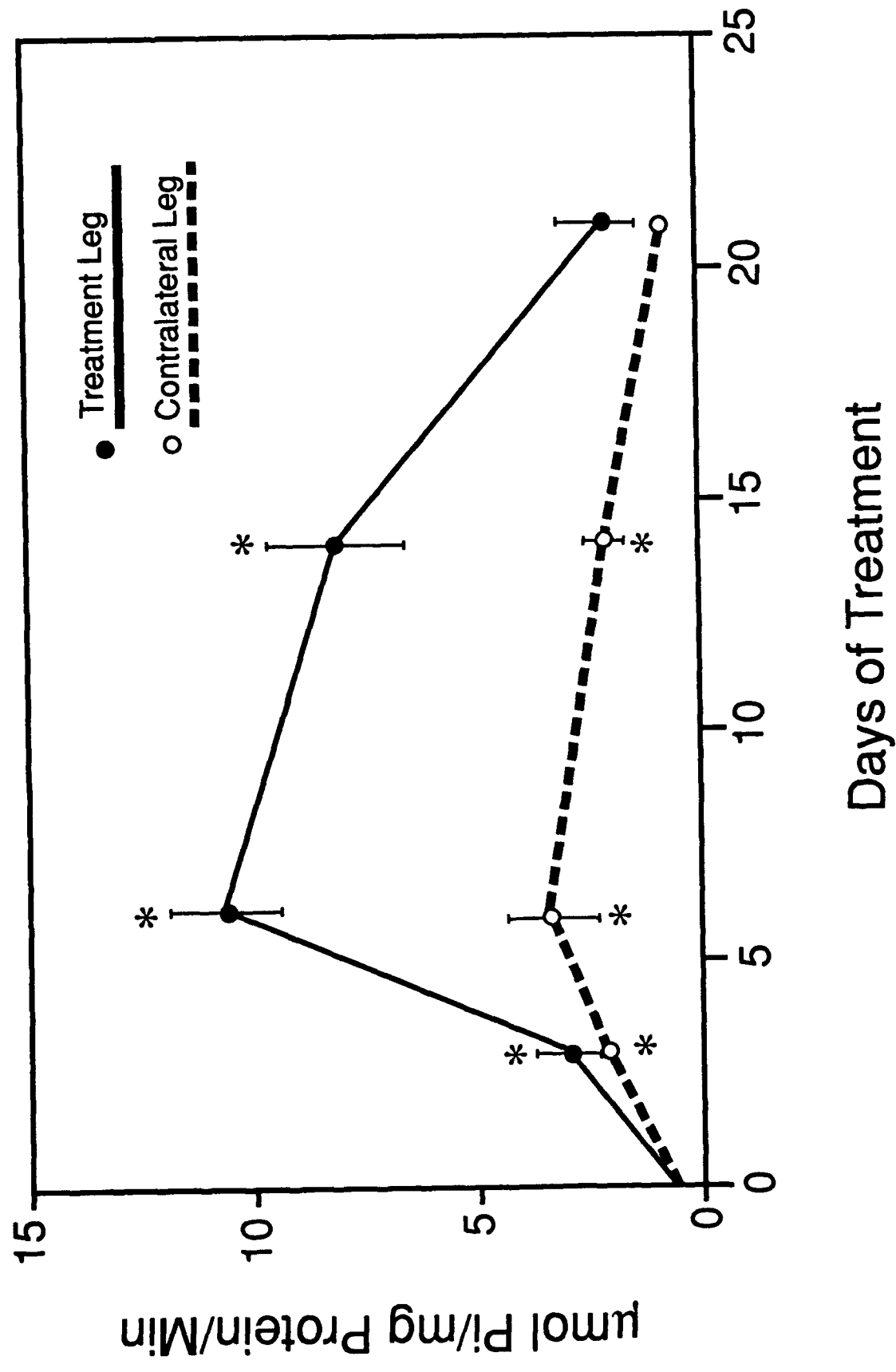
Figure 2. Specific Activity of Phospholipase A_2 in Matrix Vesicle-Enriched Membranes (MVEM) Isolated from Healing Endosteal Bone following Marrow Ablation.

MVEMs were isolated by differential centrifugation of homogenates of newly forming bone in the treated and contralateral tibias. Values are the mean $\pm$ SEM for three samples; each sample represents the pooled tissue of six treatment or contralateral legs. *p ~ 0.05 for each time point compared to time 0. Activity was determined as the amount of arachidonate hydrolized from phosphatidylethanolamine as a percent of the total arachidonate.

FIGURE 2. PHOSPHOLIPASE A$_2$
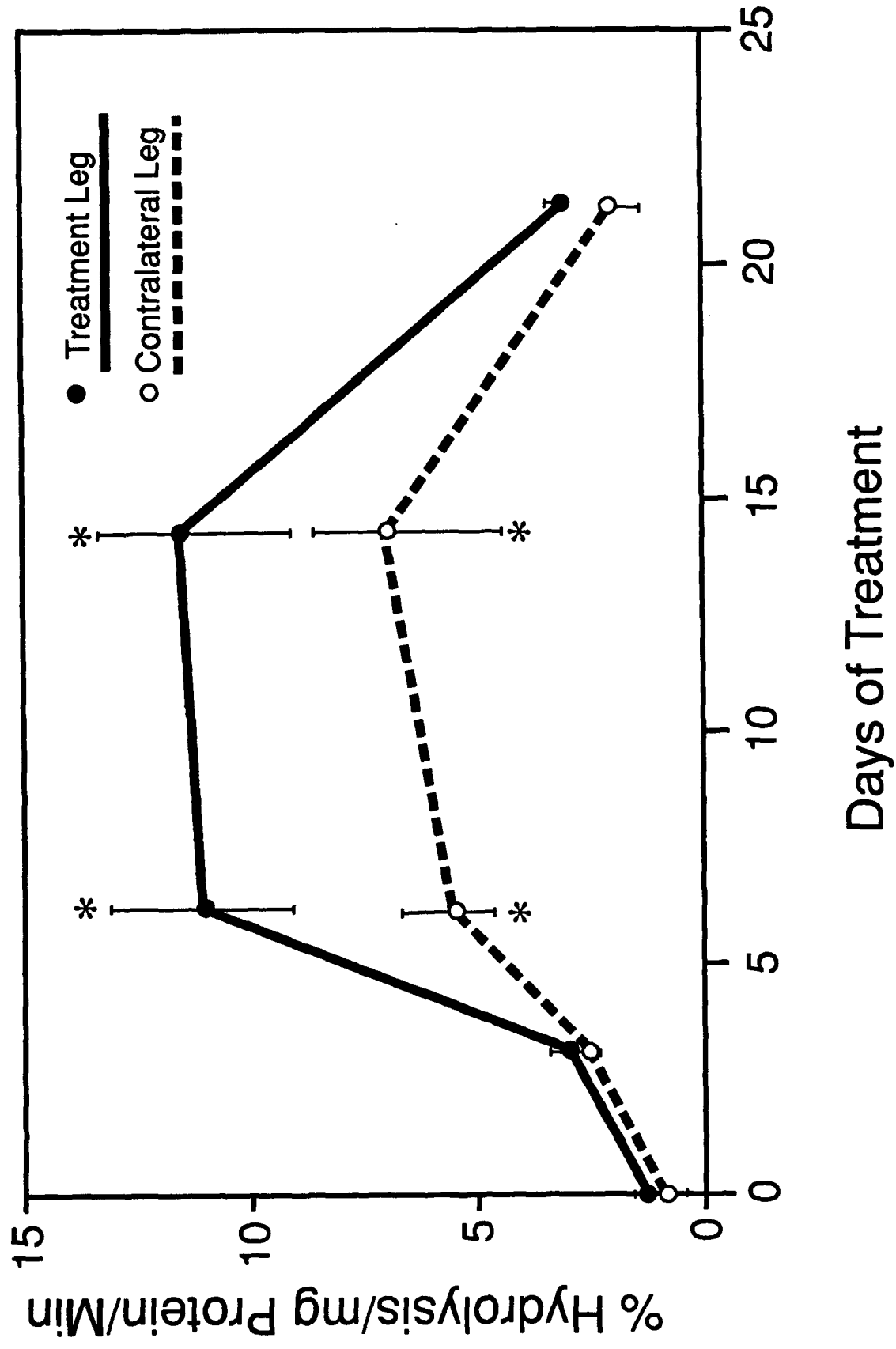
Figure 3. Distribution of Phospholipids in Matrix Vesicle-Enriched Membranes (MVEM) Isolated from Healing Endosteal Bone following Marrow Ablation (Treatment Leg).

Phospholipids were separated by HPTLC and quantitated by densitometric scan. (Sphingomyelin - SPH, Phosphatidylcholine - PC, Phosphatidylserine - PS, Cardiolipin - CL, phosphatidyl-ethanolamine - PE). Values represent means $\pm$ SEM for each phospholipid, calculated on a per-leg basis. N = 3, where each sample represents the pooled tissue from six legs.

FIGURE 3. Treatment Leg
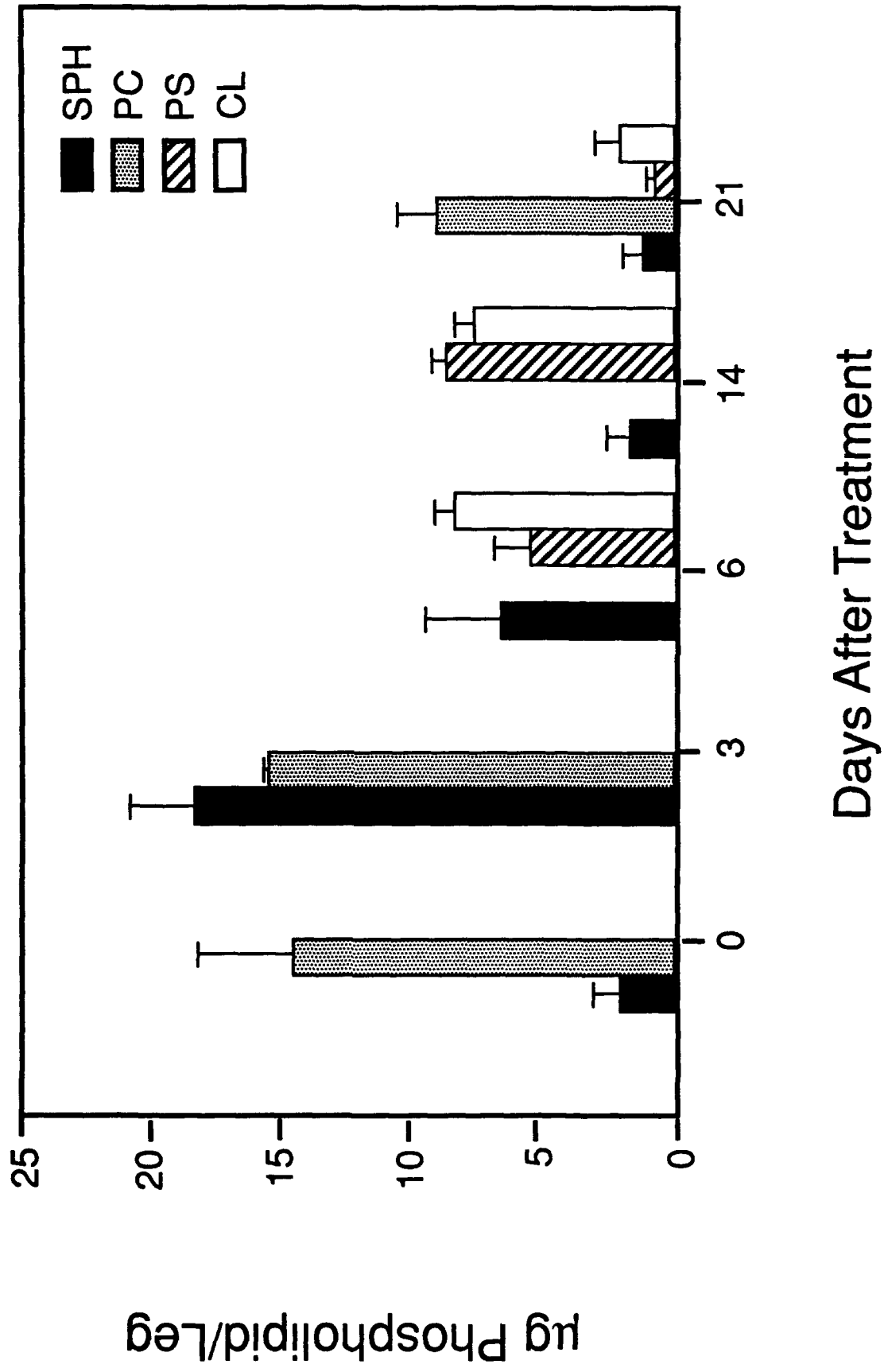
Figure 4. Distribution of Sphingomyelin (SPH), Phosphatidylcholine (PC), Phosphatidylserine (PS), Cardiolipin (CL), and phosphatidylethanolamine (PE) in Matrix Vesicle-Enriched Membranes (MVEM) Isolated from the Tibia of the Contralateral Leg following Marrow Ablation.

Values represent means $\pm$ SEM for each phospholipid, calculated on a per-leg basis. N = 3, where each sample represents the pooled tissue from six legs.

FIGURE 4. Contralateral Leg
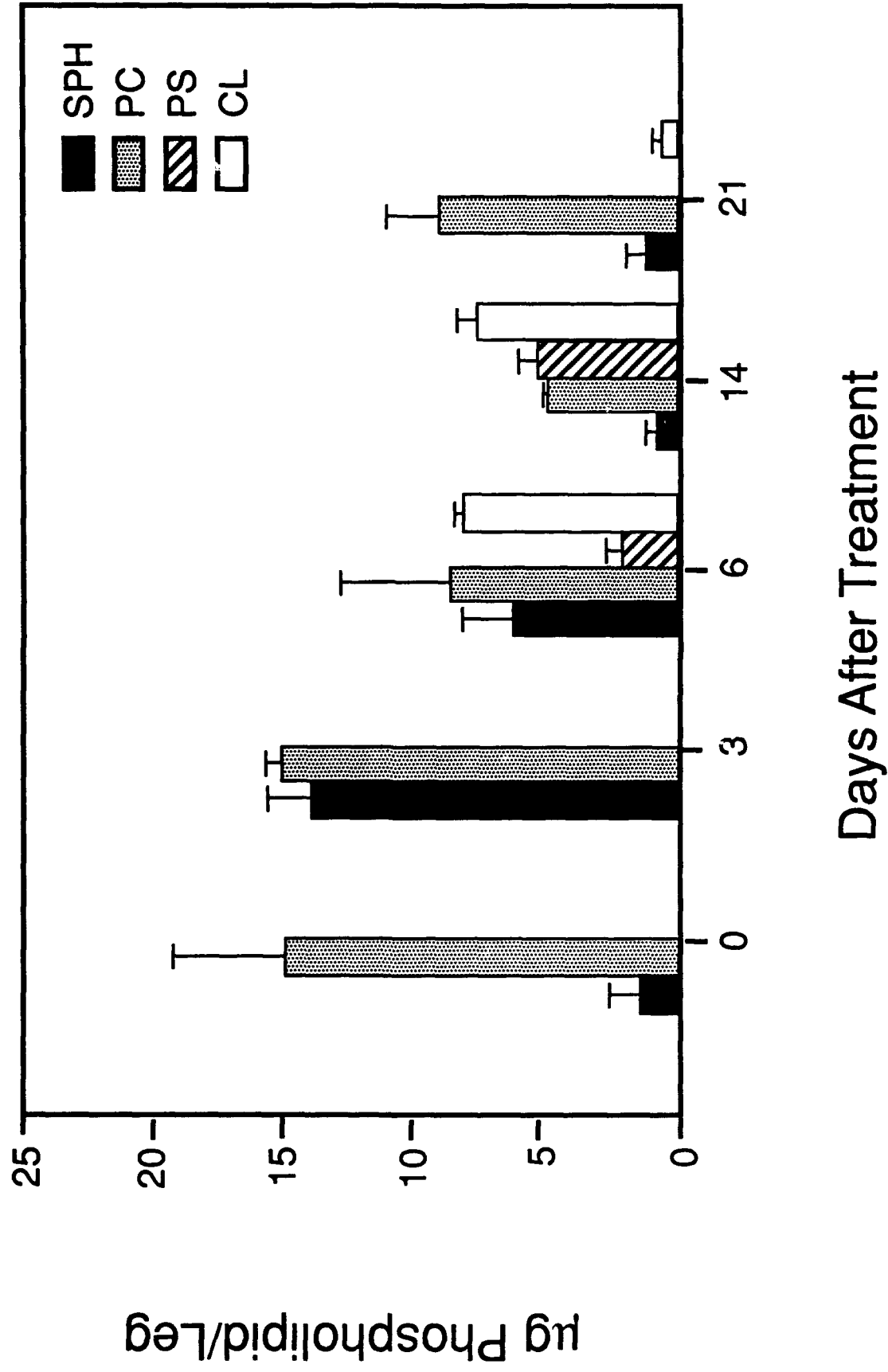
Figure 5. Change in the Phosphatidylserine (PS) Content of Matrix Vesicle-Enriched Membranes (MVEMs) Isolated from Healing Bone following Marrow Ablation of Treatment and Contralateral Tibias.

Values represent means $\pm$ SEM for each phospholipid, calculated on a per-leg basis. N = 3, where each sample represents the pooled tissue from six legs. Data at days 6 and ?4 are statistically significant (p < 0.05) when compared to time 0 and days 6 and 21.

FIGURE 5. PHOSPHATIDYLSERINE LEG
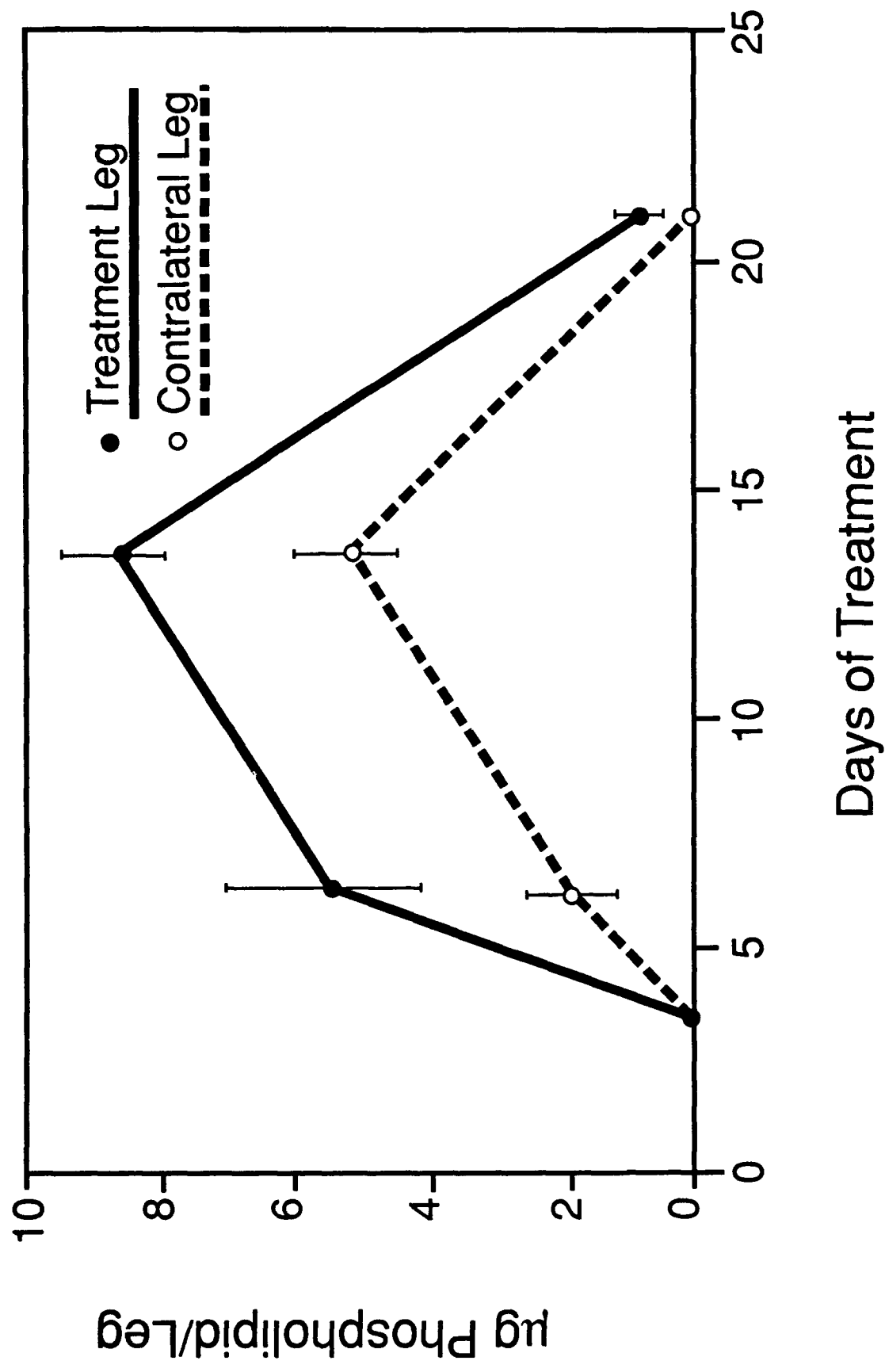
III. METHODS AND MATERIALS

IMPLANTS

Four glass-ceramic materials were surgically placed: two osseointegrating (KG-Cera and Mina-13) and two non-osseointegrating. The chemical composition of the materials in weight percent are shown on Table 1. The production of these materials has been described previously (Gross and Strunz, 1985). Glass blocks of these compositions were crystallized by heating and cooling, using a time scheme not revealed by the manufacturer. Before insertion, the implant materials were sawed into rectangular blocks (1.1 X 1.1 X 2mm), cleaned by ultrasonication, and sterilized by dry heat at 140°C for 90 minutes (Gross et al., 1981).

EXPERIMENTAL MODEL

Six hundred twelve rats of the Hebrew University (Sabra) strain, weighing 400g each, were used in the experiment. The rats were divided into five groups. Thirty-six animals were placed in a nonsurgical control group (time 0--no surgery was performed on either leg prior to marrow collection), while the remaining 576 animals (144/group: KG-Cera, Mina-13, KGy-213, M 8/1) were equally distributed in the surgical model.

In the animals receiving implants, infrapatellar surgical incisions were performed under Ketanest/Rompun anesthesia (25mg--5mg/Kg body weight) to achieve access to the proximal aspect of the right tibia (no surgery was performed on the contralateral tibia prior to marrow collection). Entrance to the marrow chamber was achieved by frontal penetration of the antero-

medial aspect of the exposed bone with a saline-cooled, round dental burr (#4) and a 20,000 RPM motor. The bone marrow chamber was evacuated by repeated washings with saline through a canula introduced into the intrabony space. Following ablation, individual samples of the glass-ceramic materials were introduced into the right tibia marrow space through the previously mentioned portal, followed by surgical closure of the access site.

The animals of each test group were further subdivided into groups of 36, representing harvest days 3, 6, 14, and 21. At harvest, bilateral tibial excisions were performed on both experimental and control animals (day 0) under Ketanest/Rompun anesthesia (25mg--5mg/Kg body weight). The contents of each marrow space were carefully evacuated by curettage under a constant flow of saline and placed in individual containers.

MATRIX VESICLE PREPARATION

Matrix vesicle-enriched microsomes (MVEM) were prepared according to the method of Watkins et al. (1980) and Cyboron et al. (1982). This method has been shown by Register et al. (1986) to yield MVEMs which support *in vitro* hydroxyapatite formation, whereas, enzymatically isolated matrix vesicles often do not. In brief, the pooled samples were homogenized using a polytron for two one-minute bursts, separated by a one-minute cooling period, at 0°C in 5ml 50mM N-Tris, 1.5mM $MgCl_3$, and 10% w/w sucrose, pH 7.5. Homogenates were centrifuged at 500g for ten minutes using the Beckman Model TJ-6 tabletop centrifuge (Beckman, Palo Alto, CA) to pellet any cells. The supernatant was collected and then

centrifuged in a Beckman Ultracentrifuge, model L5-65B (Beckman, Palo Alto, CA) for 20 minutes at 14,460g to pellet a mitochondria/membrane fraction. Subsequent to collection of the supernatant, it was centrifuged at 84,700g for 45 minutes to pellet the MVEM fraction. This pellet was resuspended in 10% sucrose, carefully layered over a sucrose step-gradient composed of 10%/40% sucrose in a Tris-MgCl$_3$ buffer, and centrifuged at 85,700g for 60 minutes. Following ultracentrifugation, the material at the interface was collected, resuspended in 10% sucrose, and once again carefully placed on a 10%/40% sucrose gradient with the Tris-MgCl$_2$ buffer. The tubes were once again ultracentrifuged at 85,700g for one hour to pellet the MVEM. The resulting MVEMs were resuspended in 1ml each 0.9% NaCl and used for the biochemical analyses. Protein content was determined by the methods outlined by Lowry et al. (1951).

ENZYME ASSAYS

Alkaline Phosphatase. Alkaline phosphatase (orthophosphoric monoester phosphohydrolase alkaline [E.C. 3.1.3.1]) was measured as the release of para-nitrophenol from para-nitrophenylphosphate at pH 10.2 (Bretaudiere and Spillmann, 1984) and specific activity determined. The procedure was divided into two parts (cold-active/heat-deactivated), in that alkaline phosphatase from blood is more susceptible to heat deactivation (Bretaudiere and Spillman, 1984). One-half of the sample to be tested was placed in a hot Water Bath Shaker (American Optical, South Bridge, MA) set at 56°C for 30 minutes. Fifty μl of each sample was placed in each of two wells of a 96-well plate, both for the active and

deactivated samples. 50μl of AMP buffer was added to each well to initiate the color reaction. The plates were then sealed with a sheet of parafilm and placed in a warm room and allowed to react. Following the color change, 100μl of NaOH was placed in each well to stop the reaction. The plate was then placed in a BioRad EIA Reader Model 2550 (Richmond, CA) and read at 405nm. The results of the duplicated samples were averaged.

Phospholipase A_2. Phospholipase A_2 (E.C. 3.1.1.4) was measured as a function of hydrolysis of ^{14}C-arachidonate from phosphatidylethanolamine-L-2-4-palmitoyl-2-arachidonyl-R^{14}C (PEC) (New England Nuclear) (Collard-Torquebiau, 1987). This portion of the experiment was performed according to the specifications outlined by Newkirk and Waite (1971). 50μl of PEC (18,000 CPM) was sonicated in an ice bath for 30 seconds, interrupted for 60 seconds, and followed by an additional 30-second sonication period. The suspension was added to the standard incubation mixture containing 2mM $CaCl_2$, 0.2M NaCl, 100mM Tris-HCl, pH 8.5, and 400μl of sample. Samples and the control were incubated in a Shaking Water Bath (American Optical, South Bridge, MA) at 37°C for 60 minutes. The reactions were stopped by the addition of 5ml of chloroform:methanol 2:1 (v:v) and vortexed. The solutions were allowed to stand overnight at 4°C.

The aqueous (upper) and lipid phases (lower) were separated, followed by concentration of the lipids using N_2. The lipids were redissolved in 50μl chloroform:methanol 2:1 (v:v) and then spotted on one-dimensional, high performance, thin-layer chromatography (HPTLC) (Analtech - Newark, NJ) (Dugan, 1985).

Prior to spotting, the preformed silica gel plates were washed with chloroform:methanol 1:1 (v:v), then with absolute ethanol: chloroform: 50mM amonium hydroxide 50:6:6 (v:v:v) and allowed to dry. The purpose for prewashing the plates is to eliminate hydrophilic and hydrophobic contaminants from the surface. After spotting the samples and controls, the phospholipids were separated using a mobile phase of ethanol:chloroform:amonium hydroxide 50:6:6 (v:v:v). Chromatograms were developed by exposure to iodine vapors. Following development, the plates were removed from the tank and allowed to dry. Two standards were also run on each plate: a) PEC and the standard incubation mixture without MVEM and b) arachidonate. Material co-migrating with PEC or arachidonic acid was removed from the thin-layer chromatogram and counted by liquid scintillation spectroscopy (Beckman LS-1701, Palo Alto, CA). The phospholipase A_2 activity was measures as the percent hydrolysis of ^{14}C-arachidonate from the total ^{14}C-PEC and expressed as specific activity.

INT-Reductase. Succinate-2-(para-iodophenyl)-3-para-(nitro-phenyl)-5-phenyl-tetrazolium-reductase (INT-reductase) activity was assayed as a measure of mitochondrial contamination according to Morre, 1971; Pennington, 1961; and Glaser and Conrad, 1981. Briefly, 100μl of the MVEM suspension, containing 0.2-0.5μg protein, was added to 0.9ml substrate (5mM potassium phosphate, 0.11% [w:v]) 2-[para-iodo-phenyl]-3-[para-nitrophenyl]-5-phenyl-tetrazolium [INT], 55mM sodium succinate, 25mM sucrose) and incubated for 15 minutes at 37°C. The reaction was stopped with 1ml 10% (w:v) trichloroacetic acid (TCA). The reduced dye was

extracted with 4ml of ethyl-acetate in stoppered glass tubes and read at 490nm on a Gilford 2600 Optical Reader (Gilford, Oberlin, OH). The concentration of the reduced dye was used to assess activity as μmol/mg protein/minute.

Phospholipid Composition. The relative phospholipid composition of the MVEM was determined using a modification of the HPTLC methodology previously described for phospholipase A_2 analysis (Schwartz et al., 1990c; Dugan, 1985), the exception being the elimination of iodine for visualization. Samples of each MVEM suspension (300μl) were extracted with 2ml of chloroform:methanol (2:1, v:v), and the resultant lower phases collected, dried under N_2, and redissolved in 50μl of chloroform:methanol (2:1, v:v). Following prewashing and drying of the HPTLC plate, the entire sample was spotted, the chromatograms developed, and finally sprayed with cupric sulfate. Phospholipids were rendered visible by heating the cupric sulfate saturated plates for ten minutes at 170°C and quantified by enhanced laser densitometry (Ultrascan XL, LBK Bromma, Bromma, Sweden). The concentration of each phospholipid was determined by comparison to standards run on the same plate and to standard concentration gradients established by the use of comparable plates run at the same time. Data are expressed per leg as a point of reference, since all MVEM per leg were used in the initial suspension from which the phospholipids were extracted. Phospholipid standards were bovine brain L-alpha-phosphatidyl-inositol, L-alpha-phosphatidylserine, L-alpha-phosphatidyl-choline, sphingomyelin, bovine heart cardiolipin, and *E. coli* L-

alpha-phosphatidyethanolamine (Sigma Chemical Co., St. Louis, MO). All solvents were reagent grade. Chloroform was redistilled before use.

Statistical Analysis. Mean and standard error of the mean (SEM) were calculated for each parameter. The differences among groups were determined using analysis of variance; differences between groups were assessed using the Student's t-test. Statistical significance was set up at $p < 0.05$.

Purpose. This experiment is part of an extensive study dealing with primary mineralization at the interface of known osseointegrating and non-osseointegrating implant materials. The experiment utilizes assays of extracellular matrix vesicle enzymes and lipid specific activity to biochemically ascertain the ability of these materials to integrate with bone (Schwartz et al., 1990b). It is felt that the biochemical analysis of the enzyme activity and lipid composition can be a valuable adjunctive test for bone formation, mineralization, and the physiological response of mineralized tissue to different implant materials. Utilization of these procedures can significantly aid in the selection of materials for clinical application. In addition to studying the direct biochemical impact of osseointegrating and non-osseointegrating implant materials on mineralizing tissues, systemic biochemical activity was also evaluated by comparing the biochemical assays of the contralateral tibia to those seen in the treatment leg.

Table 1: Composition of glass ceramic implants

IMPLANT COMPOSITION
(in weight percent)

	Bone-Bonding		Non-Bonding	
	KG Cera	Mina 13	KGy-213	M 8/1
SiO_2	46.2	46	38	50
$Ca(PO_3)_2$	25.5	16	13.5	7.1
CaO	20.2	33	31	-
Na_2O	4.8	-	4	5
MgO	2.9	5	-	-
K_2O	0.4	-	-	-
Al_2O_3	-	-	7	1.5
Ta_2O_5	-	-	5.5	-
TiO_2	-	-	1	-
B_2O_3	-	-	-	4
$Al(PO_3)_3$	-	-	-	2.4
SrO	-	-	-	20
La_2O_3	-	-	-	6
Gd_2O_3	-	-	-	4

(Data are adapted from Blumenthal et al., J. Biomedical
Materials Research 22:1033-1041, 1988.)

IV. RESULTS

MATRIX VESICLE-ENRICHED MICROSOMES (MVEM)

The enrichment of the n pellet with matrix vesicles was verified on the basis of alkaline phosphatase specific activity. The MVEM had the transmission electron microscopic appearance typical of matrix vesicles. Because no efforts were made to retain mineral during processing, all matrix vesicles appear empty or amorphous (data not shown).

There was no mitochondrial contamination of the MVEM fraction. INT-reductase specific activity in the MVEM fraction was comparable to that of matrix vesicles produced by growth zone chondrocytes in culture (Table 2) and was at least 1/10 that of the mitochondrial/membrane fraction for which it is a marker enzyme. These data are comparable to those published by Glaser and Conrad (1981) for chick epiphyseal cartilage fractions. During tibial healing, there was no change in INT-reductase in the MVEM fraction from any of the treated or contralateral limbs.

ALKALINE PHOSPHATASE

Matrix vesicle alkaline phosphatase specific activity was sensitive to the nature of the adjacent implant material (Figure 6). Alkaline phosphatase specific activity in matrix vesicles from KG Cera-implanted tissue increased significantly by the third day post-injury (Figure 6A). Enzyme activity peaked by day 6, declined slowly through day 14, and, although still elevated at day 21, was approaching the nonsurgical control values (day 0). Similarly, but at a lesser magnitude, matrix vesicle

Table 2: Succinic-p-iodonitrotetrazolium violet (int) reductase activity in matrix vesicle-enriched membranes

		Specific Activity μMol/μg Protein/Minute	
Implant	Day of Healing	Treatment Leg	Control Leg
KGy-Cera	0	2.6 $\pm$ 0.9	2.9 $\pm$ 0.5
	3	4.4 $\pm$ 1.5	3.1 $\pm$ 0.9
	6	3.5 $\pm$ 1.2	3.4 $\pm$ 1.9
	14	3.5 $\pm$ 0.7	2.0 $\pm$ 0.3
	21	1.8 $\pm$ 0.4	2.4 $\pm$ 0.5
KGy-213	0	2.6 $\pm$ 0.3	2.9 $\pm$ 0.1
	3	1.5 $\pm$ 0.5	2.2 $\pm$ 0.4
	6	2.8 $\pm$ 0.5	3.4 $\pm$ 1.1
	14	3.9 $\pm$ 1.0	3.4 $\pm$ 0.9
	21	4.1 $\pm$ 0.9	2.9 $\pm$ 0.9
Mina	0	2.6 $\pm$ 0.3	2.9 $\pm$ 0.1
	3	4.2 $\pm$ 1.2	1.6 $\pm$ 0.6
	6	2.8 $\pm$ 1.1	4.0 $\pm$ 1.1
	14	2.4 $\pm$ 1.0	1.8 $\pm$ 0.7
	21	4.4 $\pm$ 1.4	3.1 $\pm$ 0.8
M-8	0	2.6 $\pm$ 0.3	2.9 $\pm$ 0.1
	3	3.6 $\pm$ 1.4	2.4 $\pm$ 0.2
	6	3.2 $\pm$ 0.8	1.9 $\pm$ 0.4
	14	1.7 $\pm$ 0.3	2.9 $\pm$ 0.4
	21	2.6 $\pm$ 0.7	1.6 $\pm$ 0.5

Growth Zone Chondrocytes

Matrix vesicles	3.9 $\pm$ 1.7
Mitochondria/ membranes	44.8 $\pm$ 6.7

Figure 6. Alkaline Phosphatase Specific Activity of Matrix
Vesicle-Enriched Membranes (MVEM) following Marrow Ablation from
Treatment and Contralateral Tibias which Have Been Implanted with
Osseointegrating and Non-Osseointegrating Implant Materials.

MVEMs were isolated by differential centrifugation of
homogenates of newly forming bone in the treated and
contralateral tibias. Each sample represents the pooled tissue
of six treatment or contralateral leg. *p ~ 0.05 for each time
point compared to time 0.

alkaline phosphatase increased in Mina 13-implanted legs (Figure 6C) by day 3, peaking at day 6, and gradually decreasing through day 21 to that of the control.

Matrix vesicle alkaline phosphatase activity in tissue adjacent to the nonintegrating implants did show some increase over the time zero control values, but the magnitude of the increase was much smaller than seen in the osseointegrating tissues. In the KGy-213 sample (Figure 6B), maximum stimulation was less than half that observed in the KG Cera samples; in addition, maximum stimulation was delayed until day 14, with a decline to near control by day 21. In tissue adjacent to M 8/1 implants (Figure 6D), MVEM alkaline phosphatase activity peaked at day 6 post implant, showed a marked depression of both the treatment and control legs on day 14, and a slight elevation of values on day 21.

In addition, the data revealed differences in MVEM alkaline phosphatase activity in response to the type of osseointegrating or non-osseointegrating implants used (Figure 6). At all time points, enzyme activity was greatest in matrix vesicles from KG Cera-treated tissue when compared to those from Mina 13-treated tissue. At early time points, response to KGy-213 and M 8/1 were comparable, but at days 14 and 21, the enzyme activity in the KGy-213 samples remained elevated above that of the time zero control bone, whereas, in the M 8/1 bone, it had returned to baseline.

Implant-specific responses were observed in the contralateral limbs as well. MVEM isolated from the endosteal

bone in the contralateral tibia of KG Cera-implanted animals (Figure 6A) exhibited changes in alkaline phosphatase activity that mimicked those in the treated limb, but at a lower magnitude. There also was an increase in MVEM alkaline phosphatase in the contralateral limb of animals implanted with Mina 13 (Figure 6C), which plateaued at day 6 and remained constant until day 21. At days 14 and 21, the levels of enzyme activity were the same in the treated and contralateral limbs. Changes in MVEM enzyme activity in the contralateral limb of animals with KGy-213 implants (Figure 6B) were identical to those seen in the treated limbs; the same was true of the contralateral limb of animals with M 8/1 implants (Figure 6D). Peak activity in all contralateral samples and in the non-integrating tissues was consistently less than $15 \mu mol$ Pi/mg protein/minute. However, peak activity in the tissue adjacent to osseointegrating implants was greater than $15 \mu mol$ Pi/mg protein/minute and in the KG C a-treated tissues specifically, greater than $30 \mu mol$ Pi/mg protein/minute.

PHOSPHOLIPASE A$_2$

MVEM phospholipase A$_2$ activity was also sensitive to the presence of implants. In the limbs implanted with KG Cera (Figure 7A), MVEM phospholipase A$_2$ activity was maximal at day 6. By day 14, the activity had begun to decrease and by day 21 was not significantly different from the control. Activity in limbs with Mina 13 implants were also increased at day 6, but peak activity was not observed until day 14 (Figure 7C). By day 21,

Figure 7. Specific Activity of Phospholipase A_2 of Matrix
Vesicle-Enriched Membranes (MVEM) following Marrow Ablation from
Treatment and Contralateral Tibias which Have Been Implanted with
Osseointegrating and Non-Osseointegrating Implant Materials.

MVEMs were isolated by differential centrifugation of
homogenates of newly forming bone in the treated and
contralateral tibias. Each sample represents the pooled tissue
of six treatment or contralateral leg. *p $\leq$ 0.05 for each time
point compared to time 0. Activity was determined as the amount
of arachidonate hydrolyzed from phosphatidylethanolamine as a
percent of the total arachidonate.

FIGURE 7. PHOSPHOLIPASE A$_2$
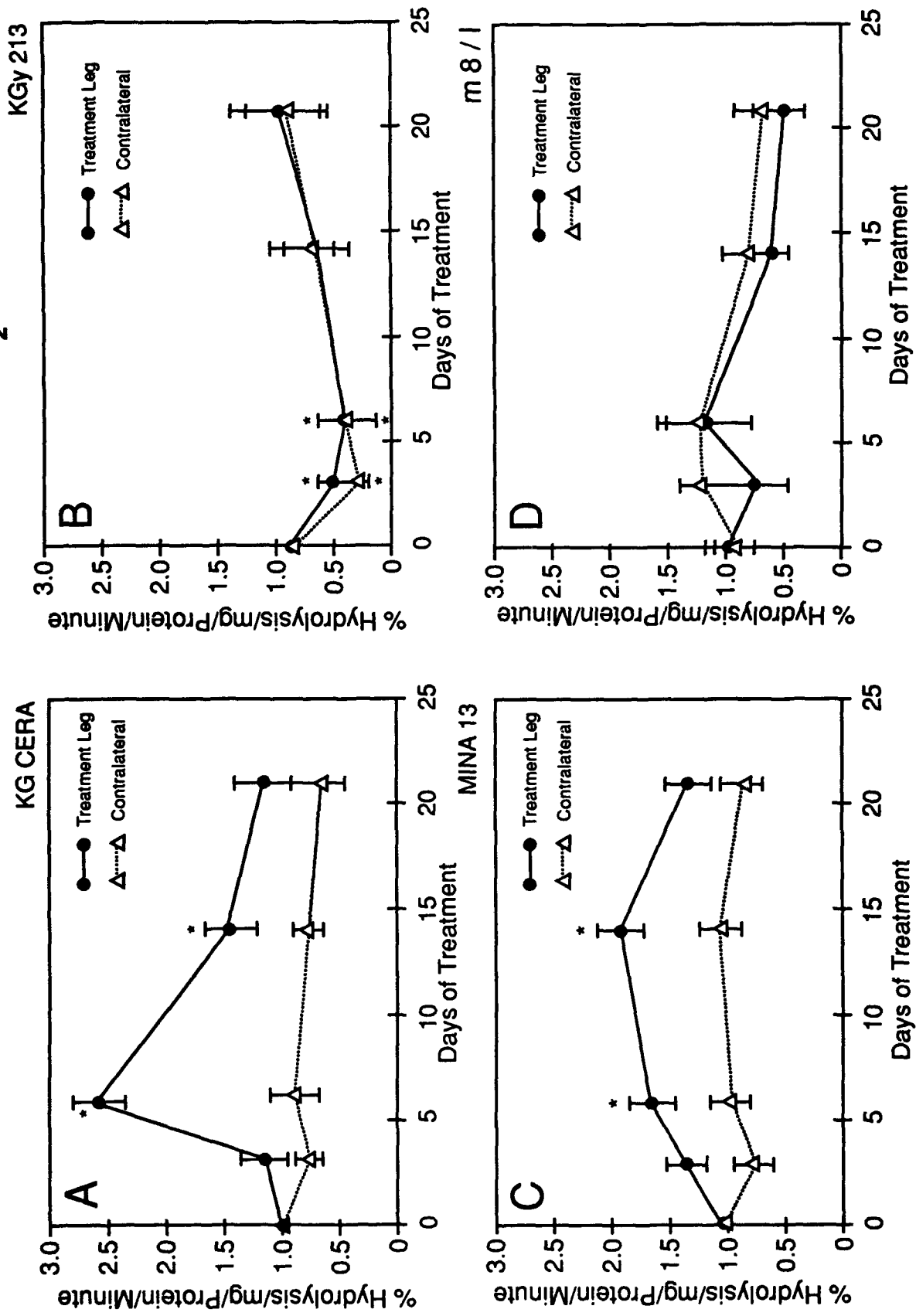
activity was once again not significantly different from the control. The greatest stimulation of phospholipase A_2 activity was observed in matrix vesicles isolated from tissue adjacent to the KG Cera implant. At day 14, enzyme activity was comparable in matrix vesicles from tissues adjacent to both of the osseointegrating implants. Unlike MVEM from osseointegrated tissues, the MVEM from the nonintegrating tissues either exhibited inhibition in the phospholipase A_2 at days 3 and 6 (KGY-213) or had an observable depression on days 3, 14, and 21 (M 8/1) (Figures 7B and 7D).

No changes were observed in the phospholipase A_2 activity of MVEM isolated from the contralateral limbs of animals with osseointegrating implants (Figures 7A and 7C). Once again, the contralateral limb activity mimicked that of the treatment limb, but at significantly reduced levels. Phospholipase A_2 activity in MVEM from the contralateral limbs of animals with non-integrating implants was identical statistically to that of the treated limbs (Figures 7B and 7D). This included the suppression of enzyme activity observed at days 3 and 6 in KGy-213 samples (Figure 7B), and the elevation in days 3, 14, and 21 of M 8/1.

PHOSPHOLIPID COMPOSITION

MVEM phospholipid composition was also affected by the nature of the implant used (Table 8). With all of the implants, a time-dependent fluctuation in the content of sphingomyelin was observed. While the peak concentration of sphingomyelin varied between days 6 and 14, there was no pattern that distinguished between osseointegrating and nonintegrating implants. Similarly,

MVEM from tissue adjacent to all implants exhibited a decrease in phosphatidylcholine content.   Phosphatidylcholine continued to

Figure 8. Change in the Phosphatidylserine (PS) Content of Matrix Vesicle-Enriched Membranes (MVEM) following Marrow Ablation from Treatment and Contralateral Tibias which Have Been Implanted with Osseointegrating and Non-Osseointegrating Implant Materials.

Each sample represents the pooled tissue *from six legs*. Data at days 6 and 14 are statistically significant (p < 0.05) when compared to time 0 and days 6 and 21.

FIGURE 8. PHOSPHATIDYLSERINE
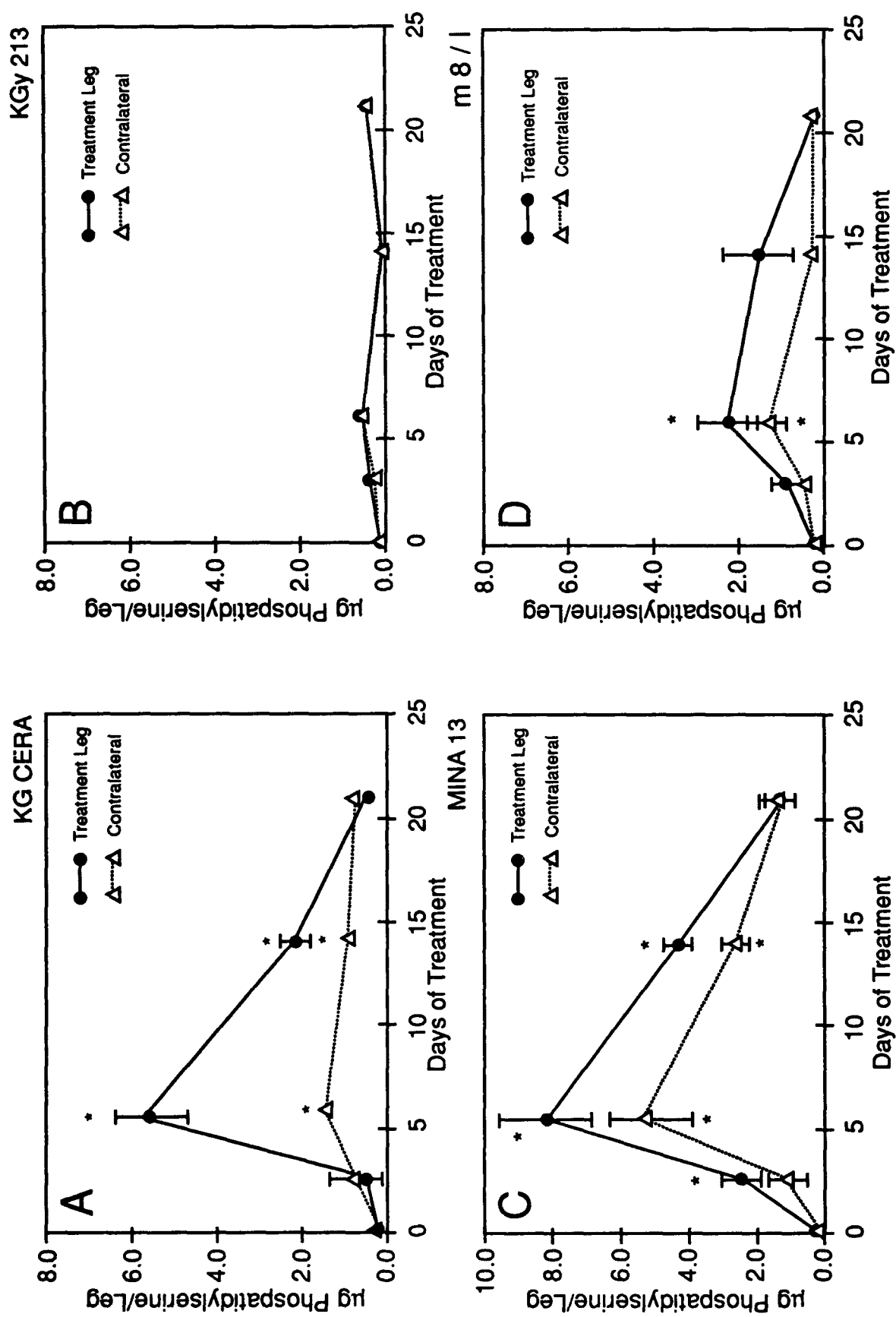
Table 3: **The effect of glass ceramic implant on phospholipid content**

Cera-Implant
Treatment Leg
μg Phospholipid/Leg

Day of Healing	Sph	PC	PS	CL
0	2.8 ± 1.3	12.4 ± 3.0	0.3 ± 0.1	1.1 ± 0.3
3	3.4 ± 0.3	17.1 ± 3.3	0.4 ± 0.4	0.6 ± 0.1
6	8.8 ± 3.2	2.4 ± 0.7	5.8 ± 1.0	4.2 ± 0.6
14	11.3 ± 5.0	3.4 ± 3.3	2.2 ± 0.4	3.9 ± 0.1
21	1.0 ± 0.2	4.5 ± 1.3	0.3 ± 0.1	2.9 ± 0.5

KGy 213-Implant

Treatment Leg
μg Phospholipid/Leg

Day of Healing	Sph	PC	PS	CL
0	2.8 ± 1.3	12.4 ± 5.0	0.3 ± 0.1	1.1 ± 0.3
3	3.8 ± 1.2	6.0 ± 2.0	0.3 ± 0.1	2.5 ± 0.2
6	4.1 ± 1.8	3.5 ± 1.5	0.4 ± 0.1	1.8 ± 0.4
14	8.7 ± 0.5	1.6 ± 0.9	0.0 ± 0.0	0.1 ± 0.0
21	3.9 ± 0.7	9.6 ± 0.5	0.3 ± 0.1	2.2 ± 0.4

MINA-Implant
Treatment Leg
μg Phospholipid/Leg

Day of Healing	Sph	PC	PS	CL
0	2.8 ± 1.3	12.4 ± 3.0	0.3 ± 0.1	1.1 ± 0.3
3	8.7 ± 4.2	8.6 ± 1.4	2.4 ± 0.3	3.2 ± 0.3
6	11.4 ± 4.7	5.2 ± 2.6	8.2 ± 1.3	10.1 ± 0.3
14	4.8 ± 1.3	2.6 ± 1.5	4.3 ± 0.4	2.7 ± 0.3
21	2.0 ± 0.8	0.0 ± 0.0	1.1 ± 0.4	0.6 ± 0.3

M8-Implant
Treatment Leg
μg Phospholipid/Leg

Day of Healing	Sph	PC	PS	CL
0	2.8 ± 1.3	12.4 ± 5.0	0.3 ± 0.1	1.1 ± 0.3
3	2.7 ± 1.6	2.4 ± 1.2	0.8 ± 0.5	1.4 ± 0.1
6	4.2 ± 1.0	0.0 ± 0.0	2.3 ± 0.7	8.7 ± 2.7
14	13.6 ± 1.6	0.0 ± 0.0	1.5 ± 0.8	5.8 ± 0.5
21	1.0 ± 0.6	8.4 ± 1.7	0.1 ± 0.1	1.3 ± 0.2

**Table 4:   THE EFFECT OF GLASS CERAMIC IMPLANT
ON PHOSPHOLIPID CONTENT**

Cera-Implant
Contralateral Leg
μg Phospholipid/Leg

Day of Healing	SPH	PC	PS	CL
0	1.8 ± 0.6	8.5 ± 2.2	1.9 ± 0.6	1.6 ± 0.3
3	3.8 ± 2.5	14.2 ± 4.3	1.2 ± 1.1	2.3 ± 0.3
6	2.0 ± 1.0	7.6 ± 1.5	1.0 ± 1.0	5.2 ± 1.0
14	6.9 ± 1.9	2.9 ± 1.3	0.9 ± 0.8	3.0 ± 1.2
21	2.9 ± 1.5	15.5 ± 4.1	0.6 ± 0.5	1.3 ± 0.3

KGy 213-Implant
Contralateral Leg
μg Phospholipid/Leg

Day of Healing	SPH	PC	PS	CL
0	1.8 ± 0.6	8.5 ± 2.2	1.9 ± 0.6	1.6 ± 0.3
3	17.2 ± 7.5	6.1 ± 2.8	0.0 ± 0.0	1.3 ± 0.4
6	13.3 ± 1.3	0.0 ± 0.0	0.0 ± 0.0	4.9 ± 0.6
14	7.1 ± 0.3	0.0 ± 0.0	0.0 ± 0.0	3.0 ± 0.7
21	5.6 ± 0.9	4.2 ± 0.9	0.3 ± 0.2	1.6 ± 0.5

MINA-Implant
Contralateral Leg
μg Phospholipid/Leg

Day of Healing	SPH	PC	PS	CL
0	1.8 ± 0.6	8.5 ± 2.2	1.9 ± 0.6	1.6 ± 0.3
3	0.0 ± 0.0	4.3 ± 0.8	5.0 ± 0.6	4.3 ± 0.7
6	0.7 ± 0.4	2.3 ± 1.5	4.4 ± 1.0	4.9 ± 0.9
14	0.5 ± 0.4	2.9 ± 1.8	7.5 ± 1.8	6.0 ± 0.7
21	1.9 ± 1.7	7.9 ± 2.7	1.6 ± 1.5	3.4 ± 0.7

M8-Implant
Contralateral Leg
μg Phospholipid/Leg

Day of Healing	SPH	PC	PS	CL
0	1.8 ± 0.6	8.5 ± 2.2	1.9 ± 0.6	1.6 ± 0.3
3	0.2 ± 0.2	17.1 ± 3.2	0.4 ± 0.3	1.8 ± 0.3
6	1.3 ± 0.5	7.3 ± 3.5	1.6 ± 1.0	1.7 ± 0.5
14	15.7 ± 2.9	0.0 ± 0.0	0.2 ± 0.1	6.4 ± 1.5
21	3.9 ± 0.8	0.0 ± 0.0	0.0 ± 0.0	1.1 ± 0.2

decrease throughout the time course in MVEM from integrating tissue, whereas, in the nonintegrating samples, this phospholipid began to return to baseline levels by day 21. Cardiolipin content exhibited a time-dependent increase in MVEM from all tissues examined. Peak concentrations were observed at day 6.

The most dramatic effects of the implant materials were associated with phosphatidylserine content (Figure 8, Table 3). At day 6, there was a 23-fold increase in phosphatidylserine content in MVEM isolated from tissue adjacent to KG Cera (Figure 8A) and a 33-fold increase in MVEM from Mina 13-implanted tissue (Figure 8C). Levels were reduced at day 14, but still elevated above the control; however, by day 21, phosphatidylserine content had returned to control levels. No change in phosphatidylserine was noted in MVEM from KGy-213-implanted tissue (Figure 8B), where there appeared to be a relative absence of the phospholipid. While there was a nine-fold increase in MVEM phosphatidylserine content in M 8/1 implanted tissue at day 6, by day 14 there was no significant difference between the treatment leg and the control (Figure 8D).

The phospholipid content of MVEM isolated from the contralateral legs was altered as well (Figure 8, Table 4). However, the effects of implantation were not necessarily identical in the implanted and contralateral limbs. For example, sphingomyelin content increased in MVEM from all samples except those from Mina 13-implanted animals. The magnitude of increase and the time of peak concentration varied, depending upon the type of implant the animal had. Furthermore, the variation did

not correlate with whether the implants were osseointegrating or nonintegrating. Fluctuations in phosphatidylcholine were comparably variable. In contrast, in all samples examined, MVEM cardiolipin content displayed a biphasic increase, peaking between days 6 and 14. Changes in phosphatidylserine content in all of the contralateral limbs mirrored the changes in the control limb, but at a reduced magnitude.

The effects of the osseointegrating and nonintegrating implants on MVEM phosphatidylserine content are displayed graphically for comparison in Figure 3. In MVEM isolated from both KG Cera- (Figure 8A) and Mina 13- (Figure 8C) implanted limbs, phosphatidyleserine content was increased maximally at day 6. There was an increase in the contralateral limb as well, but its magnitude was less than in the treated leg and was dependent on the type of implant used: Mina 13 > KG Cera. MVEM phosphatidylserine in the nonintegrating tissue exhibited a truncated increase in the M 8.1 samples only; there was no change at all in the KG Cera samples. MVEM phosphatidylserine content in the contralateral limbs exhibited similar responses.

V. DISCUSSION

Although there are numerous histological studies concerning the interface between glass-ceramic implants and their tissue bed, little information is available about the biochemical response of the host at the cellular level. The marrow ablation model used in this paper has been used previously to make direct ultrastructural assessments of the events following implantation (Schwartz et al., 1990b). It has been seen that implants in general alter healing and systemic response to foreign objects. Cytomorphometric measurements of the healing endosteal bone adjacent to the four ceramics used in the present study indicate that osseointegrating and nonintegrating implants generate distinctly different responses in the production and maturation of matrix vesicles. The number of matrix vesicles associated with osseointegrated implant materials was significantly higher than those associated with the nonosseointegrating implant materials. In conjunction with these findings, a larger proportion of late-stage (crystal and rupture) matrix vesicles were associated with the osseointegrating implant materials (Schwartz et al., 1990b). The presence of the implants of varying composition alter matrix vesicle number and maturity in a material-specific manner. Finally, implant-specific responses can be detected in matrix vesicles isolated from sites distal to and not related to the primary site of injury.

Transmission electron microscope analysis of the interface tissue (Schwartz et al., 1990b) indicated that matrix vesicle maturation (the percent of each vesicle type, the vesicle

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diameter, and the distance of the vesicle from the calcification front) was delayed in tissues adjacent to all of the implant materials examined, regardless of whether they were osseointegrating or nonintegrating, the effect being greatest next to the nonintegrating implants. When the data in the present study are compared to those obtained in studies of normal healing endosteal bone following marrow ablation (Schwartz et al., 1990a), it is clear that the presence of implants inhibits MVEM enzyme activity, both in the treated and contralateral limbs. While enzyme activity in MVEMs isolated from osseointegrating tissue is enhanced over that of its contralateral limb, the effect of nonintegrating implants is to abrogate matrix vesicle activity in the treated limb so that it is identical to that of the contralateral limb. Furthermore, only the osseointegrating materials support increased numbers of matrix vesicles in the newly formed bone adjacent to the implant (Schwartz et al., 1990b). These observations can be interpreted to mean that the cells adjacent to osseointegrating implants are able to compensate for deficiencies in the matrix vesicle structure or function by increasing the number of matrix vesicles in the region.

Changes in MVEM alkaline phosphatase specific activity highlight the importance of this enzyme to the mineralization/ integration process. In tissue adjacent to integrating implants, MVEM alkaline phosphatase specific activity behaves like that of normal healing tissue in the absence of any implant (Schwartz et al., 1990a; Bab et al., 1983), with some important differences.

In the limbs receiving KG Cera implants, the increase in MVEM enzyme activity was greater than that of normally healing bone within the first three days, but behaved like normal bone at all subsequent points. Similarly, changes in MVEM alkaline phosphatase from Mina 13 samples behaved more like those in normally healing bone at the early time points, but did not show the same degree of stimulation as seen in normal bone. There was only minimal stimulation of the enzyme in the tissue adjacent to the nonintegrating implants.

Changes in MVEM alkaline phosphatase in the contralateral limbs following implantation of integrating materials were not only depressed with respect to the treated limbs, but also exhibited a delay in maturation following marrow ablation (Schwartz et al., 1990a; Bab et al., 1983). In addition, the effect of nonintegrating implants on MVEM alkaline phosphatase activity was identical in both the treatment and contralateral limbs. The literature states that there are three potential tissue responses to an implant material: 1) the material may be considered a foreign body and actively rejected; 2) the material may be recognized as foreign and be considered inert with no tissue reaction; 3) there may be varying levels of accommodation, with final acceptance of the implant material (Linder et al., 1983). According to the present experiment, there was a positive response in both the treatment and contralateral limbs of all the materials tested. Further studies of materials other than glass-ceramics should be performed to ascertain if a tissue-inert material truly exists. When compared to normal healing bone

(Schwartz et al., 1989) or the contralateral limb following marrow ablation, neither the osseointegrating nor the non-osseointegrating implants could be considered inert (Hench and Hench, 1985).

The systemic effect of marrow ablation was sensitive to the presence of implants. Implant-specific systemic responses were noted for each implant material. It is possible that dissolution of the implant surface could result in release of ions into circulation which might directly affect sites distal to the implant. Trace metal analyses were not performed, so this possibility cannot be ruled out. It is more likely, however, that selective adsorption of serum factors onto the implant material could change the initial immune response to the injury, with systemic distribution of the wound healing response. Changes in the healing process could also alter the systemic effects due to altered release of various regulatory substances. Regardless of the cause, the data demonstrate clearly that bone cells respond to the stimulus in an implant-specific manner at sites distal to the treated tissue.

Alterations in MVEM phospholipase A_2 activity provide a biochemical clue for understanding the mechanism of delayed matrix vesicle maturation in response to implants. During normal bone healing, MVEM phospholipase A_2 exhibits a 12-fold increase in activity by day 6, which remains constant until day 14, when it returns to baseline (Figure 8). During this same time period, there was both an increase in amorphous as well as crystal-laden matrix vesicles, indicating that mineral deposition was occurring

(Figure 8). In contrast, the increase in phospholipase A_2 activity in MVEMs isolated from osseointegrating tissue is never as great as that seen in normal healing bone (Figures 2 and 7), nor does it remain elevated in bone isolated from KG Cera-implanted tissue after 14 days. Peak phospholipase A_2 activity was not reached in the Mina 13-implanted tissues until day 14, albeit at very low levels. In tissue adjacent to nonintegrating implants, there was either no increase in phospholipase A_2 activity or it was inhibited during the critical time period.

It has been hypothesized that phospholipase A_2 activity is needed for production of lyso-phospholipids. Its role is felt to be in the disruption of the matrix vesicle membrane during the "rupture" phase of development by altering the phospholipid composition, content and fluidity (Wuthier et al., 1978). These substances are associated with loss of matrix vesicle membrane integrity and subsequent exposure of the hydroxyapatite crystals to the extracellular environment. Assuming that the hydroxy-apatite crystals formed within the vesicle are needed as a nidus for bulk phase mineral deposition, failure of the matrix vesicles to rupture could have severe consequences for calcification and osseointegration. Enzyme activity in tissues adjacent to both of the osseointegrating implants was comparable, but somewhat depressed from that of normal healing bone (Figures 1, 2, 6, and 7). Unlike MVEM from osseointegrated tissues, the MVEM from the nonintegrating tissues either exhibited inhibition in the phospholipase A_2 or had an observable depression (Figure 7).

It has been found that phospholipase A_2 activity results in the release of arachidonic acid (Schwartz et al., 1990c), which is a substrate for prostaglandin production. Prostaglandin may act directly on the cells, matrix vesicles, or both to alter membrane activity. Related studies in our lab have shown that alkaline phosphatase activity in cultures of osteoblast-like osteosarcoma cells is inhibited by indomethacin, a specific inhibitor of prostaglandin production (data not shown). In this scenario, the prostaglandin functions as a second messenger. Failure to detect any change in the MVEM phospholipase A_2 activity in the contralateral limbs could be related to changes in the type of regulatory factors released following injury and implantation. It is important to note that the inhibition of phospholipase A_2 observed in limbs with KGy-213 implants was also observed in the contralateral limbs.

If ability to promote osseointegration is related to matrix vesicle-dependent hydroxyapatite formation, it is not surprising that MVEM phosphatidylserine content was affected. This phospholipid is normally enriched in matrix vesicles (Schwartz et al., 1990a; Boyan et al., 1988; Peress et al., 1974) and is intimately associated with mineral formation as the principle organic constituent of calcium-phospholipid-phosphate complexes (CPLX) (Boskey and Posner, 1977) and the principle phospholipid moiety of calcifying proteolipids (Boyan, 1991). When it is complexed with mineral, it is resistant to phospholipase activity (Boyan, unpublished), explaining its elevated content at day 6 in normally healing marrow-ablated bone (Schwartz et al., 1990a)

(Figure 8) and bone adjacent to osseointegrating implants (Figure 8). The relative absence of MVEM isolated from nonintegrating tissue or their contralateral limbs suggests that these matrix vesicles have failed to form mineral-complexed lipids (Figure 8).

The phospholipid data presented in this thesis are expressed in absolute amounts rather than in relative percent compositions. Thus, it is possible to assess the real changes in the content of any individual lipid and not only its fluctuation in terms of the other phospholipids. Therefore, the changes in phosphatidylcholine are real, and not an artifact. The results indicate that matrix vesicle production is continuous in time; that is, new matrix vesicles are produced throughout the healing process, since phosphatidylcholine content returns to baseline values in the nonintegrating tissues at day 21, following failure of the tissue to calcify. In contrast, MVEM phosphatidylcholine content in the integrating tissue is significantly reduced at day 21, corresponding with rupture of the matrix vesicles and mineralization of the matrix. Fluctuations of phosphatidylcholine in the contralateral limbs does not appear to be related to events in the treated tissue, and their significance is difficult to assess.

High cardiolipin and phosphatidylethanolamine content are a feature of matrix vesicles isolated from cartilage (Peress et al., 1977) and chondrocyte cultures (Boyan et al., 1988). The increase in this phospholipid around day 6 may reflect a burst in production of matrix vesicles predestined to serve as calcification initiation sites. Similarly, sphingomyelin has

also been shown to be a constituent of matrix vesicles. The increase in this phospholipid between days 6 and 14 also suggests new matrix vesicle production. Another interpretation of the data is that multiple types of matrix vesicles are produced, each with a specific set of functions. The results of this experiment demonstrate, for the first time, that biochemical changes occurring at the cell/implant interface are directly related to the production or suppression of primary mineralization.

Regulation of matrix vesicle structure and function has been shown in chondrocyte (Boyan et al., 1991) and osteoblast (Boyan et al., 1990; Bonewald et al., 1991) cultures in response to a number of endocrine, autocrine, and paracrine regulatory factors. The results of this paper indicate that matrix vesicle structure and function can be regulated during the bone formation process *in vivo* as well. The inhibition of matrix vesicle enzyme activity observed in response to ceramic implant materials may be due to the direct effects of ions leaching from the implant surface (Pappas and Cohen, 1968; Blumenthal et al., 1988). Matrix vesicles are discrete organelles with enzymatic activity and phospholipid metabolism that are distinct from that of the cell (Schwartz et al., 1990c). It is also possible that these ions alter cell activity, with subsequent effects on matrix vesicle production, including new gene transcription, protein synthesis and vesicle formation.

This study also clearly demonstrates the problems inherent in using contralateral limbs as controls for studying implant efficacy and toxicity. Contralateral limbs exhibited changes in

MVEM structure and function that reflected those in the limbs with nonintegrating implants, although at lower magnitude, comparable to the changes observed following simple marrow ablation (Schwartz et al., 1990a). However, the response to nonintegrating materials was much different, treated limbs and contralateral limbs behaving in an identical fashion, at least with respect to alkaline phosphatase activity. The issues were further complicated by analysis of the phospholipid content of the matrix vesicles. In this instance, the effects on the contralateral limbs were distinct from those of the treated limbs, indicating that the choice of parameter is critical in assessing the toxicity of a particular material.

VI. <u>SUMMARY</u>

Osseointegrating and nonosseointegrating implants have been shown to have a profound effect on the biochemical activities of extracellular matrix vesicles, particularly in respect to their participation in primary mineralization. Previous TEM and computer-generated morphometric analytic studies have shown a reduction in maturation and differentiation of matrix vesicles associated with all implant materials. However, in the osseointegrated implant materials, compensation was noted in the number of matrix vesicles produced, which rendered a healing curve comparable to that of normal healing bone. To examine the role that osseointegrating and nonintegrating implants have on the biochemistry during primary mineralization, two enzymes (alkaline phosphatase and phospholipase A_2) and six lipids (bovine brain L-alpha-phosphatidylinositol, L-alpha-phosphatidyl-serine, L-alpha-phosphatidylcholine, sphingomyelin, bovine heart cardiolipin, and *E. coli* L-alpha-phosphatidyethanolamine) were evaluated as to content and activity. Osseointegrating implant materials (KG-Cera, MINA-13) stimulated alkaline phosphatase activity and, to a lesser extent, phospholipase A_2 activity, while the nonintegrating implant materials (KGy-213 and M 8/1) either had minimal effect or depressed enzyme activity. Both of these enzymes are directly related to the mineralization process. Failure to activate these enzymes will block the mineralization process and result in a fibrous union at the implant site. Neither primary nor secondary mineralization would be expected following implantation of nonbonding implant materials. The

initiation of primary mineralization is an expected consequence following insertion of the osseointegrating implant materials. The results of this experiment demonstrate, for the first time, that biochemical changes occur at the cell/implant interface which are directly related to the process of primary mineral formation in tissues adjacent to osseointegrating and non-integrating materials. Of particular interest, and potentially of great significance to the field of medical implants, is that all of the implant materials studied produced systemic effects distal to and not directly involved with the primary site of injury. The changes paralleled those of the injured limb only at a reduced magnitude. The consequence of the reaffirmation of this finding is that there are potential positive and negative side effects of all implants placed in the body. Finally, the experimental model as outlined could be a valuable adjunctive diagnostic tool for establishing the clinical efficacy of existing and future implant materials.

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VIII. VITA

Thomas Scott Marshall was born on August 2, 1953, to Joseph John and Ruth Elaine Marshall in Bangor, Maine. The son of a career US Air Force officer, he had the opportunity to experience several overseas and continental assignments prior to his father's retirement from active duty in 1966. Following graduation from Port Huron High School in Port Huron, Michigan, in 1971, he attended Michigan Technological University. In 1975, he received a Bachelor of Science degree in Biology and was commissioned a second lieutenant in the United States Air Force. Selected as an Air Force Institute of Technology student, he enrolled in the University of Michigan School of Dentistry in 1975 and received the degree of Doctor of Dental Science in August, 1979. Following graduation, he embarked on a career with the US Air Force, with stations at Rhein-Main AB, Germany; Hickam AFB, Hawaii; Suwon AB, Korea; and Kelly AFB, Texas. In July 1989, he entered the Postdoctoral Prosthodontics program at the University of Texas Health Science Center in San Antonio in conjunction with Wilford Hall USAF Medical Center. He is presently assigned to Wilford Hall Medical Center as a resident in Prosthodontics, with an anticipated graduation date of June 1992. Upon completion of training, he anticipates continuing his career and service with the US Air Force.

PUBLICATIONS

1. Modulation of Matrix Vesicle Enzyme Activity and Phosphatidylserine Content by Ceramic Implant Materials During Endosteal Bone Healing. Submitted for publication 1992: Calcified Tissue International.

2. Matrix Vesicle Enzyme Activity and Phospholipid Content in Endosteal Bone Following Implantation of Bonding and Nonbonding Implant Materials. Published in 1991: Clinical Oral Implants Research 2:112-120.

Manuscripts

1. Response of Bone Forming Cells to Ceramic Surfaces *In Vivo* and *In Vitro*. Manuscript for oral presentation 1990: American Academy of Orthopaedic Surgeons.

Abstracts

1. The Effects of Bone Bonding and Non-Bonding Implants cn Matrix Vesicle Alkaline Phosphatase Activity during Tibial Bone Healing. Abstract for oral presentation submitted 1990: Annual Meeting, Orthopaedic Research Society.

Poster Presentations

1. Glass Ceramic Implants Affect Matrix Vesicles during Bone Healing. Poster Presentation 1991: International Association of Dental Research.

2. The Effects of Bone Bonding and Non-Bonding Implants on Primary Mineralization. Poster Presentation 1991: American Society for Bone and Mineral Research.

John J. Sharry Research Competition

1. A Biochemical Analysis of Osseointegrating and Non-Osseointegrating Implant Materials during Primary Mineralization. Oral Presentation 1991: Third Place Finalist - John J. Sharry Prosthodontic Research Competition.